SUBJECT: Construct Hazard Analysis for J-18-44

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# I. INTRODUCTION

A TSCA Microbial Commercial Activity Notice (MCAN) has been received from the National Institute of Standards and Technology (NIST) for an intergeneric yeast strain called *Saccharomyces cerevisiae* NE095 (NIST ERCC 00095). This fungus has been genetically modified by stably inserting a functional *S. cerevisiae URA3* gene and a 438 bp DNA sequence found in the extremophile *Methanocaldococcus jannaschii* into Chromosome IV.

The subject microorganism *S. cerevisiae* NE095 will be used as a qualitative and quantitative material for microbial abundance measurements. The submitter also notes that development and characterization of the subject microorganism has been supported through Interagency Agreements between the Department of Homeland Security (DHS) Science and Technology Directorate and NIST.

*S. cerevisiae* NE095 will be produced at a manufacture site not controlled by the submitter, quantified by the submitter, and made available as a Reference Material (RM) through the NIST Office of Reference Materials. NIST RMs are homogeneous, stable materials characterized for a reference value (total cells per vial) and sold at cost. As a NIST RM, *S. cerevisiae* NE095 will be lyophilized as a whole cell material (both live and dead cells present), and stored in vials containing on the order of ~$10^7$ cells per vial. This material will also be relevant to other quantification methods, such as whole cell counting via flow cytometry, microscopy, particle counting technologies, etc.

*Saccharomyces cerevisiae* is a well-characterized eukaryotic fungus that is found worldwide. *S. cerevisiae* is a budding yeast that undergoes sexual or asexual reproduction depending on environmental conditions and is a model organism used in molecular biology studies. It is present in some foodstuffs and used in various fermentation processes. *S. cerevisiae* can be an opportunistic pathogen, but the organism is generally recognized as safe (U.S. EPA, 1997).

# II. GENETIC CONSTRUCTION OF THE MICROORGANISM

The construct of the submission microorganism is discussed in detail in the Genetic Construction Report for J-18-44 (Peñalva-Arana, 2018).

The final construct was inserted via homologous recombination into a non-coding region of Chromosome IV in the recipient microorganism BY4739. The final construct is 1645 bp in length, and consists of flanking regions from Chromosome IV of BY4739, a 1106 bp DNA sequence for the functional *S. cerevisiae URA3* gene, and the 438 bp ERCC-00095 sequence from *M. jannaschii*. This 1645 bp insert replaces 903 bp of native noncoding sequence (544595 bp - 545497 bp) in Chromosome IV.

The subject microorganism's (*S. cerevisiae NE095*) whole genome was sequenced (Illumina MiSeq) and the results verified a single copy of the inserted sequence on Chromosome IV.

# III. POTENTIAL HAZARDS POSED BY THE GENETIC MODIFICATIONS

## A. Inserted Genes

A segment of DNA (438 bp) from *Methanocaldococcus jannaschii* DSM 2661 (ERCC-00095) and the *S. cerevisiae URA3* gene encoding DNA was inserted into *S. cerevisiae* BY473 to form the subject microorganism *S. cerevisiae* NE095.

*M. jannaschii* is an extremophilic methanogen. These archaea are found in marine environments and are associated with hydrothermal vents. The inserted archaeal sequence (ERCC-00095) encodes a partial protein derived from *M. jannaschii*'s pstB (a phosphate specific complex component) involved in a phosphate transport system (Bult et al., 1996). Phosphate transport pathways are ubiquitous across organisms and in the environment. Phosphate is required for nucleic acid synthesis, as well as for cellular metabolic and transport functions. Thus, phosphate transport pathways are integral to basic cellular function.

ERCC-00095 also does not encode any transfer or mobilization functions. This sequence will be used as a specific selection marker for the subject microorganism.

In *S. cerevisiae* S288C *URA3* encodes orotidine-5'-phosphate decarboxylase, an enzyme that catalyzes the synthesis of pyrimidine ribonucleotides (Umezu et al., 1971). The recipient strain *S. cerevisiae* BY4739 lacks the *URA3* gene and cannot synthesize uracil, therefore requiring an exogenous source for growth. The insertion of a *URA3* gene into BY4739 enables the strain to grow in uracil-deficient medium. *S. cerevisiae* NE095 thus is able to grow in both typical yeast broth and synthetic defined yeast broth without uracil.

## B. Cellular Effects of Modifications of Inserted Sequences

The *M. jannaschii* sequence (ERCC-00095) insertion is unlikely to impact cellular functions of since the insertion site is non-coding and the intergeneric sequence, if expressed, is not expected to result in a functional protein. ERCC-00095 only includes 3 out of 8 open reading frames and 62 out of 252 (~25%) amino acids; therefore, ERCC-00095 is not expected to result in a functional protein in *S. cerevisiae*

NE095. Even in an unlikely event that ERCC-00095 was expressed, *S. cerevisiae* already contains a phosphate transport system, thus this intergeneric DNA insertion would not provide the subject microorganism with a new metabolic capability (Oshima et al., 1997).

*URA3* is also naturally found in the genus Saccharomyces and will serve to catalyze the synthesis of pyrimidine ribonucleotides in *S. cerevisiae* NE095 (Umezu et al., 1971). This enables the subject microorganism to be able to synthesize its own uracil and grow in uracil-deficient media, allowing for the selection of successfully transformed cells (Boeke et al., 1984; Alani et al., 1987).

Antibiotic resistance is a concern, particularly in organisms engineered for use outside of closed systems. Antibiotic resistance genes can be transferred to unrelated microorganisms in the environment through various mechanisms. The intergeneric sequence does not encode antibiotic resistance and therefore poses no risk for contributing to the pool of genes associated with resistance to antibiotics with therapeutic applications.

### C. Stability of Inserted Genes

The final inserted DNA sequence (1,645 bp) consists of the *URA3* sequence (1,106 bp), followed by the ERCC-00095 sequence (438 bp) and flanking regions from Chromosome IV of BY4739 (101 bp).

This construct was inserted via homologous recombination into a noncoding portion of BY4739 Chromosome IV. The subject microorganism (*S. cerevisiae* NE095) was verified via sequencing to have a single copy of ERCC-00095 and URA3 DNA on Chromosome IV.

Since the introduced DNA sequence was incorporated into *S. cerevisiae*'s genome (Chromosome IV), its potential transfer to the environment is very low as compared to DNA sequences kept on plasmids. The inserted DNA sequence therefore is expected to be maintained by the organism.

## IV. POTENTIAL FOR HORIZONTAL GENE TRANSFER

Horizontal gene transfer (HGT) has been well documented in yeast, where both inter-species and inter-genus events involving *Saccharomyces cerevisiae* are known to be possible (Table 1) (Fitzpatrick et al., 2012). The reported rates of yeast HGT are relatively low, but these transfer events can significantly impact niche specification, disease emergence, and shifts in metabolic capabilities.

**Table 1.** Examples of Fungal-Fungal Horizontal Gene Transfer

| Recipient | Donor | Chromosome/Gene | Reference |
|---|---|---|---|
| *Nectria haematococca* | Fungal | PEP gene cluster | Temporini & VanEtten (2004) |
| *Podospora anserina* | Fungal | Sterigmatocystin cluster | Slot & Rokas (2011) |
| *Aspergillus clavatus* | Fungal | ACE1 cluster | Khaldi et al. (2008) |
| *Aspergillus niger* | Fungal | Fumonisin cluster | Khaldi & Wolfe (2011) |
| *Saccharomyces cerevisiae* EC118 | Fungal | 34 genes | Novo et al. (2009) |
| *Aspergillus oryzae* | Fungal | Numerous functions | Khaldi & Wolfe (2008) |
| *Mycosphaerella graminicola* | Fungal | Eight chromosomes | Goodwin et al. (2011) |
| *Fusarium oxysporum* | Fungal | Four chromosomes | Ma et al. (2010) |
| *Pyrenophora tritici-repentis* | Fungal | ToxA | Friesen et al. (2006) |
| *Ceratobasidium oryzae-sativae* | Fungal | ITS | Xie et al. (2008) |

- *Modified from Fitzpatrick 2012*

Although the mechanisms of HGT in fungi are still not fully understood, some possible mechanisms have been reported involving *S. cerevisiae*. Bacteria to *S. cerevisiae* DNA transfer via conjugation was observed by Heinemann and Sprague (1989), where bacterial conjugative plasmids were used. Transformations into *S. cerevisiae* have also been reported (under specific artificial lab conditions), even though there is no known DNA uptake system for this yeast (Nevoigt et al., 2000). Bundock et al. (1995) also showed that *S. cerevisiae* was one of the first fungi shown to be susceptible to *Agrobacterium tumefaciens*-mediated transformation (ATMT).

Interspecific hybridizations between different Saccharomyces species have been shown experimentally (Marinoni et al., 1999). Industrial yeasts like *S. cerevisiae* used in fermentations, have been reported to have formed individual hybrids with *S. kudriavzevii*, *S. bayanus*, and also a triple hybrid between all three Saccharomyces species (Morales and Dujon, 2012). Beyond artificial fermentation settings, inter-species hybrids/HGT events are also known to occur in natural environments. For example, the genome of some *S. cerevisiae* strains have shown to contain DNA segments originating from *S. paradoxus*, *S. kudriavzevii*, and *S. uvarum* (Novo et al., 2009).

Inter-genus HGT between *S. cerevisiae* and *Candida glabrate* has also been reported, where plasmid transfer due to cell lysis or cytoduction were proposed as possible mechanisms (Mentel et al., 2006). Somatic fusion has also been proposed as a mechanism of whole chromosomal transfer into the ascomycota fungus *Mycosphaerella graminicola*, where 8 of its 21 chromosomes are dispensable and likely to have originated from an unknown fungal source (Goodwin et al., 2011). Anastomosis, the fusion between branches of the same or different hyphae, has also been linked to a possible HGT mechanism in certain fungal species. As filamentous fungi often utilize conidial anastomosis tubes to fuse conidia, allowing for the regulation of water, nutrients, signal molecules, nuclei, and organelles (Read et al., 2009), along with genetic material (Roca et al., 2004), there is also evidence that interspecies anastomosis between fungal pathogens has occurred (Friesen et al., 2006; Xie et al., 2008). Like other eukaryotes, ecological proximity is also thought to be associated with HGT in fungi. Novo et al. (2009) reports an example where genetic material from *Zygosaccharomyces bailii*, a major contaminant of wine fermentations, was found in the yeast wine strain *S. cerevisiae* EC118.

In fungi, most reports of HGT have been associated with a gain of osmotrophic capacity and traits of pathogenicity (Richards et al., 2006; 2011). With the ability for full genome screens, researchers have been able to further expand the fungal HGT dataset to include acquisition of genes related to the metabolism of macromolecules, amino acids, sugars, nitrogen, and nucleobases as well as the acquisition of secreted proteins and transporters (Richards et al., 2011). Other studies on fungal HGT have reported the transfer into fungi of genes involved in xenobiotic catabolism, toxin production, plant cell wall degradation, and wine fermentation (Gardiner et al., 2012; Tiburcio et al., 2010; Friesen et al., 2006; Sun et al., 2013; Garcia-Vallve et al., 2000; Novo et al., 2009). The horizontal transfer of entire metabolic gene clusters between unrelated fungi have also been discovered (Greene et al., 2014; Khaldi et al., 2008; Slot and Hibbett, 2007; Slot and Rokas, 2010; 2011; Campbell et al., 2012; Campbell et al., 2013; Patron et al., 2007; Khaldi and Wolfe, 2011). From these studies, it is noted that some pathways are more amendable to the introduction of new genes via HGT than others since metabolic networks associated with different functional categories have varying degrees of connectivity. Gene products that result in large protein complexes or interact with many partners tend to show fewer variation in copy number, which can be due to the fact that uncontrolled/unbalanced increases in gene dosage usually lead to malformed protein complexes and the accumulation of toxic intermediates in metabolic pathways (Wapinski et al., 2007; Liang et al., 2008; Sorek et al., 2007; Papp et al., 2003; Cohen et al.,

2011). Furthermore, HGT for yeasts like *S. cerevisiae*, is thought to be more advantageous for specialized metabolic pathways that are under heavy selection in highly fluctuating environments (Greene et al., 2014).

For the current submission, no transfer or mobilization functions were introduced into the subject strain, *S. cerevisiae* NE095. Since the introduced DNA sequence was incorporated into the genome (Chromosome IV), its potential transfer, to the environment and other organisms, although possible as described above, are rare compared to DNA sequences kept on mobile genetic elements (e.g. plasmids).

However, if a transfer event were to occur, the introduced sequences would likely not confer any additional metabolic capabilities to a recipient organism as no functional proteins are encoded by the intergeneric sequence ERCC-00095. As described above, ERCC-00095 only encodes for ~ 25% of *M. jannaschii*'s *pstB* protein sequence, thus is not expected to result in a functional protein in *S. cerevisiae* NE095. The organism also already has an inherent phosphate transport mechanism. *URA3* also already naturally exists and functions in wild type *S. cerevisiae*, so the risks and implications of HGT of this gene from the subject microorganism is identical to what already exists in the environment.

As mentioned previously, most known HGT events with *S. cerevisiae* and yeasts in general, have been associated with genes/gene clusters that offer the recipient organism some adaptive/fitness advantage for survival. Since the inserted DNA sequence does not offer any of these advantages, it is expected to be maintained by *S. cerevisiae* NE095 and unlikely to be transferred to other microorganisms.

# V. CONCLUSIONS

There are low hazards associated with the genetic modifications made to the recipient strain as ERCC-00095 does not encode a functional protein, and URA3 already functions in wild-type *S. cerevisiae*. Horizontal gene transfer is also unlikely since the inserted sequence offers no fitness advantage, does not introduce any novel metabolic capabilities, and also stored in Chromosome IV of *S. cerevisiae* NE095's genome.

# VI. REFERENCES

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3. Fitzpatrick, D.A. 2012. Horizontal gene transfer in fungi *FEMS Microbiol. Letters* **329**(2012)1:8.